United States
Environmental Protection
Agency

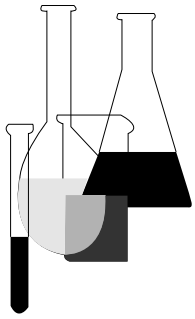
Prevention, Pesticides
and Toxic Substances
(7101)

EPA 712–C–96–347
April 1996

EPA

Ecological Effects Test Guidelines

OPPTS 850.4230
Early Seedling Growth Toxicity Test



“Public Draft”

INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Public Draft Access Information: This draft guideline is part of a series of related harmonized guidelines that need to be considered as a unit. *For copies:* These guidelines are available electronically from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines’’ or in paper by contacting the OPP Public Docket at (703) 305–5805 or by e-mail: guidelines@epamail.epa.gov.

To Submit Comments: Interested persons are invited to submit comments. By mail: Public Docket and Freedom of Information Section, Office of Pesticide Programs, Field Operations Division (7506C), Environmental Protection Agency, 401 M St. SW., Washington, DC 20460. In person: bring to: Rm. 1132, Crystal Mall #2, 1921 Jefferson Davis Highway, Arlington, VA. Comments may also be submitted electronically by sending electronic mail (e-mail) to: guidelines@epamail.epa.gov.

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on *The Federal Bulletin Board*. By modem dial 202–512–1387, telnet and ftp: fedbbs.access.gpo.gov (IP 162.140.64.19), or call 202–512–0135 for disks or paper copies. This guideline is also available electronically in ASCII and PDF (portable document format) from the EPA Public Access Gopher (gopher.epa.gov) under the heading ‘‘Environmental Test Methods and Guidelines.’’

OPPTS 850.4230 Early seedling growth toxicity test.

(a) **Scope**—(1) **Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source material used in developing this harmonized OPPTS test guideline is 40 CFR 797.2800 Early Seedling Growth Toxicity Test.

(b) **Purpose.** This guideline intended for use in developing data on the toxicity of chemical substances and mixtures (''chemicals'') subject to environmental effects test regulation. This guideline prescribes tests using commercially important terrestrial plants to develop data on the phytotoxicity of chemicals. The EPA will use data from these tests in assessing the hazard of a chemical to the environment.

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definitions also apply to this test guideline.

ECX means the experimentally derived chemical concentration that is calculated to effect X percent of the test criterion.

Germination means the resumption of active growth by an embryo.

Support media means the quartz sand or glass beads used to support the plant.

(d) **Test procedures**—(1) **Summary of the test**—(i) **Root exposure.** In preparation for the test, seeds are planted in the potting containers (or in cotton or glass-wool plugs supported in hydroponic solution) and, after germination, seedlings are thinned by pinching the stem at the support medium surface to the 10 most uniform seedlings per pot. This marks the start of the test and the time of first application of test chemical. Seedlings emerging after this time are also pinched off at the surface. Potting mixtures of sand or glass beads are subirrigated with nutrient solution. Chemicals are applied to the plants via nutrient solution or are sorbed to the support media. Plants are harvested after 14 days and analyzed for growth.

(ii) **Foliar exposure.** The foliar exposure test is identical to the root exposure test except that chemicals are applied to plants by either spraying or dusting the foliage or by exposing the plants to gas in a fumigation chamber.

(2) **Chemical application**—(i) **Root exposure.** (A) Chemicals that are soluble in water should be dissolved in the nutrient solution just prior to the beginning of the test. Deionized or glass-distilled water should be used in making stock solutions of the test chemical. Sufficient quantities

of each concentration should be made up as needed to minimize storage time and disposal volume.

(B) Chemicals that are insoluble in water, but which can be suspended in an aqueous solution by a carrier, should be added, with the carrier, to the nutrient solution. The carrier should be soluble in water, relatively nontoxic to plants, and should be used in the minimum amount required to dissolve or suspend the test chemical. There are no preferred carriers, however, acetone, gum arabic, polyethylene glycol, ethanol, and others have been used extensively in testing herbicides, plant growth regulators, fungicides, and other chemicals that affect plants. Carrier controls should be included in the experimental design of the test and tested simultaneously.

(C) Water-insoluble chemicals for which no nontoxic, water-soluble carrier is available should be dissolved in an appropriate volatile solvent. The solution should be mixed with the sand or glass beads which are then placed in a rotary vacuum apparatus and evaporated, leaving a uniform coating of chemical on the sand or beads. A weighed portion of beads should be extracted with the same organic solvent and the chemical assayed before the potting containers are filled. Solvent controls should be included in the experimental design and tested simultaneously.

(ii) **Foliar exposure.** (A) Water-soluble chemicals should be dissolved in deionized or glass-distilled water just prior to use. Sufficient quantities of each concentration should be made up as needed. These solutions should be applied daily (during a normal 5–day work week). Plants should be placed in an exhaust hood and the chemical applied to the foliage. A plastic sleeve may be fitted over the top of the pot, and the foliage sprayed with specific quantities of test solution at known concentrations. The plastic sleeve, confining the chemical to plant and pot, facilitates expression of chemical dosage to quantity per pot area (i.e., micrograms per square meter). Shoots of control plants should also be sprayed deionized or distilled water. A miniature compressed-air sprayer mounted on a pendulum and equipped to spray a plant positioned directly beneath the center of its arc of swing may be used.

(B) Water-insoluble chemicals, existing as solids, may be prepared for testing by grinding or other reduction to particles of < 200 μm diameter. Each day (during a normal 5–day work week) plants should be placed in an exhaust hood, a plastic sleeve fitted over the top of the pot, and specific quantities of chemical sprinkled uniformly over the potted seedlings. Prior to chemical application, plants should be misted with water to promote foliar retention of the chemical. Control plants should also be misted with deionized or distilled water at each treatment date and dusted with an inert material of the same particle size. Applications are expressed as quantity per unit pot area (i.e., micrograms per square meter).

(C) Chemicals existing in gaseous form at normal ambient temperatures and pressures can be generated as needed or stored under pressure. The bottled gas may be 100 percent chemical or may be mixed with an inert carrier, such as nitrogen, to known concentrations. Chemicals of controlled or measured concentrations should be metered into the exposure chamber, uniformly mixed about the plants, and exhausted through an outlet port.

(3) **Range-finding test.** (i) A range-finding test should be conducted to establish if definitive testing is necessary and to establish the concentrations of test substance used in the definitive test for each species.

(ii) The recommended procedure is to expose newly germinated seedlings to a series of widely spaced concentrations of test chemical and assess effect as growth reduction. Seeds (approximately 30) should be planted directly in containers filled to within 2.5 cm of the top with quartz sand or glass beads. If a hydroponic system is used, the seeds should be planted in plugs of cotton or glass wool supported at the top of the solution. When 50 percent of the seeds have germinated the seedlings should be thinned (by pinching) to the 10 most uniform per pot and exposed to a widely spaced concentration series (i.e., 0.01, 0.1, 1.0, 10, 100, 1,000 mg/L) of test chemical. The lowest concentration in the series, exclusive of controls, should be at the chemical's detection limit. The upper concentration, for water-soluble compounds, should be the saturation concentration. If the anticipated fate of the chemical is soil or soil water, and the mechanism of concern is root uptake, the chemical should be applied in nutrient solution to the root support media (or coated on sand or glass beads for nonwater soluble chemicals). With a chemical whose anticipated mode of exposure to plants is surface deposition by atmospheric transport, or irrigation water, the appropriate testing method may be foliar application allowing subsequent movement into the rooting zone with watering. Effect is assessed as growth reduction.

(iii) Alternatively, the seed germination/root elongation test may be used to establish the appropriate concentration range for testing.

(iv) No replicates are required and nominal concentrations are acceptable unless definitive testing is not required.

(v) Definitive testing is not necessary if the highest chemical concentration tested results in less than a 50 percent reduction in growth or if the lowest concentration tested (analytical detection limit) results in greater than a 50 percent reduction in growth.

(4) **Definitive test.** (i) The purpose of the definitive test is to determine the concentration response curves and the EC10s and EC50s for each of the species tested with the minimum amount of testing beyond the range-finding test.

(ii) At least five concentrations of chemical, exclusive of controls, should be used in the definitive test. For each species tested the concentration range should be selected to define the concentration-response curve between the EC10s and EC90s. Test chemicals should be added to the hydroponic or nutrient solution or coated on the support media for the root exposure test, or sprayed, dusted, or gassed directly on the foliage in the foliage exposure tests.

(iii) Control pots should be included in the experimental design and should be used in each run. In addition, a carrier control should also be used for those chemicals that need to be solubilized.

(iv) If plants are to be grown hydroponically, seeds should be planted in plugs of cotton or glass wool supported in the tops of the containers. When sand or glass beads are used, the recommended planting procedure is to fill the potting containers to within 2.5 cm of the top and to sow seeds directly on the support medium. After 50 percent of the seeds have germinated, the seedlings should be thinned to the 10 most uniform per pot.

(v) Alternative planting methods may be required when the chemical is highly volatile. An impervious barrier of polyethylene film, a modification of the double pot method, a glass plate, or other appropriate apparatus should be used to prevent volatilization from the root zone. Seeds should be germinated in the dark at 25 °C and seedlings with radicle lengths in the median range transplanted into the potting containers. The seedlings should be positioned such that their roots are exposed to the support media while the shoots pass through holes in the barrier. A ring of nontoxic, inert, pliable putty should be used to seal the holes around the stems. Control pots should be handled identically to the test pots except there is no exposure to the test chemical. This transplanting procedure, without the volatilization barrier, is also recommended when the test chemical is adsorbed to the support medium.

(vi) The test consists of one run for each of the recommended plant species or selected alternates. The duration of a run should be at least 14 days from the time that 50 percent of the seeds have germinated. For a particular chemical, a run is defined as exposure of the plant species to five concentrations of the chemical in a minimum of three replicate pots (10 plants per pot), with appropriate controls, followed by weight and height determinations and analysis.

(vii) All abnormalities (visible effects of the chemicals on plant growth and morphology including stunting of growth, discoloration, chlorosis and/or necrosis of the leaves, or morphological abnormalities) should be recorded. Observations of plants should be made daily (during a normal 5–day work week).

(viii) A randomized complete block design is recommended for this test with blocks delineated within the chambers or over greenhouse benches and randomization of treatment occurring within the blocks. If, because of the use of very large pots, there is inadequate space within chambers for blocking, total randomization within chambers is acceptable.

(ix) Irradiation measurements should be taken at the top of the plant canopy and the mean, plus a maximum and a minimum value, determined over the plant-growing area. These measurements should be taken daily and should be taken at least at the start and finish of the test. If the test is conducted in a greenhouse facility, hourly measurements of irradiation should be recorded and presented as daily total irradiance plus representative hourly curves for clear sky conditions and cloudy days.

(x) Temperature and humidity should be measured daily at the top of the plant canopy during each light and dark period.

(xi) Measurements of carbon dioxide concentration should be made at the top of the plant canopy (of chamber-grown plants) on a continuous basis.

(5) **Analytical measurements**—(i) **Chemical.** Stock solutions should be diluted with glass-distilled or deionized water to obtain the test solutions. Standard analytical methods, if available, should be used to establish concentrations of these solutions and should be validated before beginning the test. An analytical method is not acceptable if likely degradation products of the chemical, such as hydrolysis and oxidation products, give positive or negative interference. The pH of these solutions should also be measured prior to use.

(ii) **Numerical.** Mass and length of roots, shoots, and entire plants (root and shoot) should be measured for the definitive test. Means and standard deviations should be calculated and plotted for each treatment and control. Appropriate statistical analyses should provide a goodness-of-fit determination for the concentration-response curves.

(e) **Test conditions**—(1) **Test Species**—(i) **Selection.** (A) Test plants recommended for the definitive test include:

(*1*) *Lycopersicon esculentum* (tomato).

(*2*) *Cucumis sativus* (cucumber).

(*3*) *Lactuca sativa* (lettuce).

(*4*) *Glycine max* (soybean).

(*5*) *Brassica oleracea* (cabbage).

(*6*) *Avena sativa* (oat).

(*7*) *Lolium perenne* (perennial ryegrass).

(*8*) *Allium cepa* (common onion).

(*9*) *Daucus carota* (carrot).

(*10*) *Zea mays* (corn).

(B) Other species, of economic or ecologic importance to the region of impact, may also be appropriate and selected for testing.

(ii) **Seed selection.** Information on seed lot, the seed year or growing season collected and germination percentage should be provided by the source of the seed. Only untreated seed (not treated with fungicides, repellants, etc.) taken from the same lot, and year or season of collection should be used in a given test. In addition, all seed of a species used in a test should be of the same size class, and that size class which contains the most seed should be selected and used in a given test. Any seed which is damaged should be discarded.

(2) **Facilities**—(i) **Apparatus.** (A) Greenhouses or environmental chambers should provide adequate environmental controls to meet the carbon dioxide, humidity, irradiation, photoperiod, and temperature specifications. Chambers should be designed to prevent escape of internal air into the external environment other than through appropriate filtering material or media to prevent contamination of the external environment with the test chemical.

(B) Laboratory facilities for chemical determinations should include nonporous floor covering, absorbent bench covering with nonporous backing, and adequate disposal facilities to accommodate plant nutrient, test and wash solutions containing test chemicals at the end of each run, and any bench covering, lab clothing, or other contaminated materials.

(ii) **Containers and support media.** For each run, 18 polyethylene pots sufficiently large to grow at least 10 plants up to 14 days, are required for each species. It is equally acceptable to use small, individual containers if plants are grown in hydroponic solution. An additional three pots will be needed if a carrier control is needed. Potting containers used in each experiment should be of equal size and volume and possess the same configuration. When sand or glass beads are used, the potting containers should be filled to within 2.5 cm of their tops. Perlite, vermiculite, native soils, etc., should not be used for root support.

(iii) **Cleaning and sterilization.** (A) Potting and receiving containers, nutrient storage containers, and root support medium should be cleaned before use. All equipment should be washed according to good standard laboratory procedures to remove any residues remaining from manufacturing or prior use. Dichromate solution should not be used for cleaning beads or pots.

(B) Rooting media other than glass beads should be discarded at the end of the experiment. Disposal should conform to existing regulations.

(iv) **Nutrient media.** Half-strength modified Hoagland nutrient solution should be utilized as nutrient medium for this test. When sand or glass beads are used as support media, the potting containers should be filled with nutrient solution and drained periodically. An automated system design is recommended.

(3) **Test parameters.** Environmental conditions should be maintained as specified in this paragraph.

(i) Carbon dioxide concentration at 350 ± 50 ppm.

(ii) Relative humidity should approach 70 ± 5 percent during light periods and 90 percent during dark periods.

(iii) The level of irradiation, measured at 1 m from the source, at 350 ± 50 $\mu E/m^2$ sec at 400 to 700 nm.

(iv) Photoperiods of 16 h light/8 h darkness.

(v) Day/night temperatures at 25°/20°± 3°C.

(f) **Reporting.** Reporting requirements of 40 CFR Part 792—Good Laboratory Practice Standards apply to this guideline. The following data should be reported for each of the species tested in tabular form:

(1) Concentration of chemical in nutrient solution and in the root support material when the chemical is soluble in water or solubilized with a carrier compound, or the concentration of carrier compound in nutrient solution when carrier is used, or the quantity of chemical per unit weight of root support material when it is coated on the material.

(2) The quantity of chemical, the concentration at which it was applied, and the number of applications for those chemicals applied to the foliage.

(3) Environmental conditions (day/night temperatures, relative humidity, light intensity, carbon dioxide concentration, and photoperiod).

(4) Mass of above ground (shoot) and below ground (root) portion of each plant and mass of each whole plant (dry weight at 70 °C).

(5) Length of shoot, root, and entire plant.

(6) Visible effects of chemical, if any, on the intact plants.

(7) Means and standard deviations for mass and length of roots, shoots, and entire plants in each treatment and control. In addition, concentration- response curves with 95 percent confidence limits delineated, goodness-of-fit determination, and EC10s and EC50s identified.